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The Type-specific Substance of *Hemophilus influenzae*, Type d: the Natural Occurrence of Glucosamine Uronic Acid*

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The chemical structures of the type-specific substances of *Hemophilus influenzae* types a, b, c, e, and f have previously been studied (2–5) and all, except type e substance, have been shown to be polysugar phosphates. The type-specific substance of type d, which has previously been isolated and shown to contain nitrogen but no phosphorus (6), is the subject of the studies presented here.

The type d substance has been found, in this study, to be a highly acidic polysaccharide composed of *N*-acetylated glucosamine and glucosamine uronic acid residues. The latter sugar has previously been synthesized by Heyns and Paulsen (7); another hexosamine uronic acid, galactosamine uronic acid, synthesized by Heyns and Beck (8), has been found to be the monomer of Vi-antigen (9). However, the present paper is believed to be the first report of the natural occurrence of glucosamine uronic acid.

EXPERIMENTAL PROCEDURE

D-Glucosamine hydrochloride was obtained from California Corporation for Biochemical Research and *N*-acetylglucosamine from Nutritional Biochemicals Corporation. Galactosamine was kindly given by Dr. K. Meyer of this department.

We wish to thank Dr. K. Heyns, of Hamburg University, for the kind gift of synthetic samples of glucosamine uronic acid and galactosamine uronic acid.

Previously described methods were used for the determination of proteins (10), reducing sugars (11), hexosamines (12), hexoses (13), and uronic acids (14). Nitrogen analysis and *N*-acetyl analysis were performed by the Schwarzkopf Microanalytical Laboratories, Woodside, New York. Periodate oxidation, in 1 ml of 0.2 M acetate buffer, pH 5.0, was followed spectrophotometrically (15). Viscosity measurements were carried out in an Ostwald-Fenske microviscometer at 37°.

Paper chromatography was carried out with a descending technique on Whatman No. 1 paper in the following solvent systems: A, 1-butanol-95% ethanol-water (5:1:4 by volume); B, pyridine-1-butanol-water (50:75:150 by volume, upper layer mixed with pyridine (50 parts by volume)); C, 1-butanol-acetic acid-water (38:12:50 by volume) (16).

Paper electrophoresis was performed on Whatman No. 1 paper, by a technique similar to that described by Foster (17), under the conditions specified in the text.

Sugars were detected on paper chromatograms and electrophoretograms by development with silver nitrate-ethanolic sodium hydroxide (18), and 2-amino-2-deoxy sugars were identified by a specific spray based on the color which their deamination products give with indole-HCl (19).

Isolation of Type-specific Substance, Type d

The cells of *Hemophilus influenzae*, type d, were grown under the conditions described for type f (4) and the substance d was isolated and purified by techniques essentially similar to those used for substance e (4). The yield of lyophilized material from 100 plates was 57.5 mg.

Substance d prepared in this manner was shown to precipitate all type-specific antibody from specific antiserum, as judged by the capsular swelling and agglutination tests.

Physical Properties of Substance d

Viscosity—An aqueous solution of substance d (0.9 mg per ml) had a specific viscosity, $\eta_{sp} = 2.2$.

Mobility—Electrophoresis of a solution of substance d (concentration, 1.0 mg per ml) in 0.02 M phosphate buffer, pH 7.2, at 1° in a Tiselius cell, showed a single peak with ascending mobility of -11.83×10^{-5} cm^2 volt^{-1} sec^{-1}.

Absorption Spectra—The ultraviolet spectrum of substance d in aqueous solution (concentration, 0.75 mg per ml) showed no maxima (230 to 360 mμ) but there was an unspecific absorption, increasing rapidly below 250 mμ, which could be attributed to the turbidity of the solution.

The infrared absorption spectrum of substance d incorporated into a KBr disk is shown in Fig. 1*a*. The following assignments were made: 3.0 μ (O—H); 3.45 μ (C—H); 6.05 μ, 6.5 μ, and 7.3 μ (typical of an acetamido group); and 6.21 and 7.15 μ (O$^-$—C=O). To verify the latter assignments as the carbonyl stretching frequencies of the ion COO$^-$, the absorption spectrum of the undissociated form of substance d was measured (Fig. 1*b*). To obtain this form a solution of substance d was treated with Dowex 50-X4 (H$^+$), 50 to 100 mesh, before the lyophilization with potassium bromide and the preparation of a disk. The peak at 5.80 μ, not present in the spectrum of the dissociated form of the polysaccharide (Fig. 1*a*), was assigned to the C=O stretching frequency of COOH. The absorptions at 6.21 μ and 7.15 μ are now absent.

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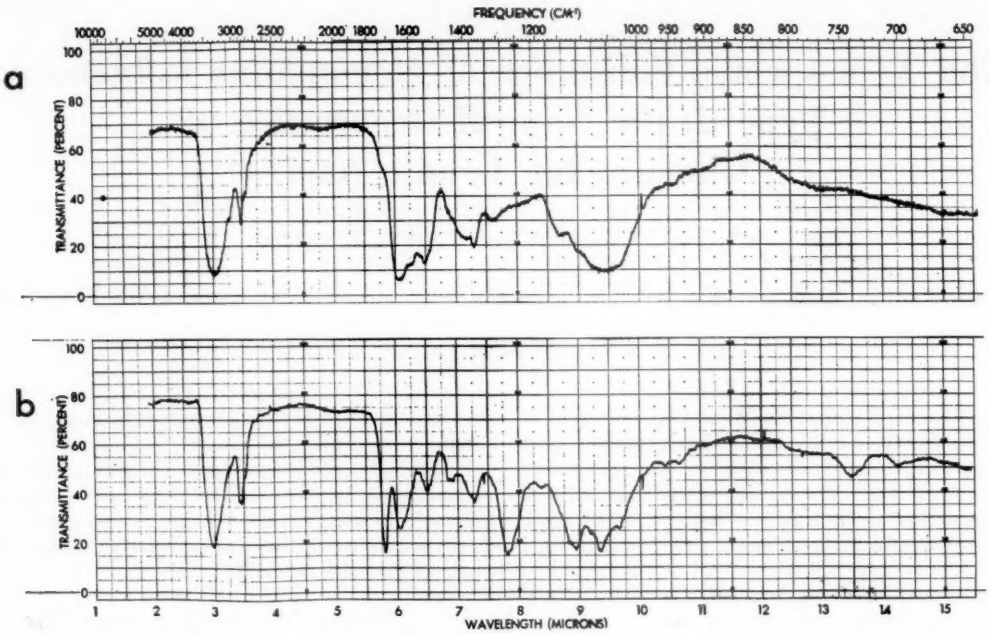

FIG. 1. Infrared absorption spectrum of type-specific substance d of *H. influenzae*; *a*, dissociated form; *b*, undissociated form.

A second new strong absorption at 7.8 μ is possibly due to C—O stretching vibrations or OH deformation vibrations.

Chemical Properties of Substance d

Elementary analysis showed that substance d contained 5.9% nitrogen (corrected for ash (7.4%) and moisture).

Substance d had a zero reducing value (11) and gave negative carbazole (14) and anthrone (13) reactions. Substance d consumed no periodate in 24 hours at room temperature. *N*-Acetyl analysis showed the presence of 16.3% acetyl groups.

Quantitative determination by the method of Lowry *et al.* (10) indicated that various preparations of substance d had protein contents ranging from 9.6% to 3.9%. A sample of substance d (3.9% protein) was treated with trypsin (twice recrystallized) in 0.1 M Tris buffer, pH 8.2, for 4 hours at 37° and recovered by precipitating twice with 3 volumes of ethanol. The protein content of this sample of substance d was reduced to 0.7% and no loss in immunological activity was observed.

Monosaccharide Units of Substance d

Hydrolysis of substance d with 2 N HCl at 100° showed a maximal liberation of 2-amino-2-deoxy sugars (12) after 6 hours, corresponding to a hexosamine content (glucosamine or galactosamine as standard) of 76.1% (corrected only for protein content). The hydrolysate was evaporated to dryness in a vacuum at 20–25°, repeatedly re-evaporated after successive additions of water, and finally the residue was dissolved in a known volume of water.

Paper chromatography of the hydrolysate in Solvent B showed two components, one at the origin and the other with a similar R_F value to glucosamine and galactosamine. In Solvent C two components with R_F values 0.12 and 0.23 were detected with the amino sugar spray reagent (19). (Markers: R_F values, glucosamine and galactosamine 0.25.)

Paper electrophoresis of the hydrolysate in 0.05 M acetate buffer, pH 4.5, at 20 volts per cm for 1½ hours, separated two components (detected with the silver nitrate spray) with migrations of +7.8 cm (major) and +9.3 cm (minor). (Markers: migrations, glucosamine, +9 cm; glucosamine uronic acid, +7.2 cm.) Electrophoresis in 0.05 M phosphate buffer, pH 8, for 2 hours at 20 volts per cm separated two components (detected with the spray for amino sugars) with migrations 0 cm and −4 cm.

Ninhydrin degradation of the amino sugar components of the acid hydrolysate and separation of the products by paper chromatography in Solvent A (20) revealed two reducing components, one remaining at the origin and the other having a similar R_F value to arabinose.

Deamination of the acid hydrolysate and separation of the resultant anhydro sugars by borate electrophoresis (19) showed products with mobilities characteristic of those from glucosamine and glucosamine uronic acid.

Selective *N*-acetylation of the hydrolysate (21) and borate electrophoresis of the mixture showed two components (*p*-anisidine-HCl spray) with migrations 2.0 cm and 12.0 cm (22 volts per cm, 1½ hours). *N*-Acetylglucosamine migrated 2.0 cm. *N*-Acetylmannosamine is easily distinguishable by its higher mobility than *N*-acetylglucosamine in this system (22).

The identity of the hexosamine uronic acid was further confirmed by chromatography on a cation exchange resin. Dowex 50-X4 (200 to 400 mesh) was washed successively with 4 N HCl, water, and 0.35 M citrate buffer, pH 5.3, and packed into a column (height, 50 cm; diameter, 1.13 cm). Aliquots (120 to 150 μg) of glucosamine uronic acid and galactosamine uronic acid were applied to the column in 2 N HCl (50 μl) and elution carried out with the citrate buffer. Fractions of 1.5 ml (flow rate, 0.23 ml per minute) were collected and analyzed by the method of Yemm and Cocking (23). Both uronic acids were eluted in sharp peaks

sufficiently separate so as not to overlap. Substance d (5.4 mg) was hydrolyzed with 2 N HCl (1 ml) at 100° for 6 hours and then an aliquot (50 μl) of the hydrolysate was chromatographed on the standardized column as described above. The peak volume (36 ml) of the uknown hexosamine uronic acid was very similar to that of glucosamine uronic acid (35 ml) but distinct from that of galactosamine uronic acid (45 ml). The hexosamine was also eluted from the column and the content of each peak was determined. The molar ratio of glucosamine uronic acid to glucosamine was 1.86.

Substance d (20 mg) was hydrolyzed with 2 N HCl (2 ml) as described above. The hydrolysate was evaporated to dryness and repeatedly evaporated with water (twice) and methanol (four times) to remove excess hydrochloric acid. Finally, the residue was dissolved in a small volume of methanol and the theoretical quantity of methanolic sodium hydroxide was added to neutralize the hydrochlorides (based on 80% amino sugar content of substance d). A flocculent precipitate resulted and was removed by centrifugation. Recrystallization of this precipitate from concentrated HCl yielded a small amount of a white microcrystalline hydrochloride which showed the same bands in its infrared spectrum as synthetic glucosamine uronic acid hydrochloride (16).

DISCUSSION

Substance d is a highly acidic polysaccharide which migrates as a single electrophoretic peak with a mobility of the order of those quoted for hyaluronic acid samples (-8.0×10^{-5} cm^2 volt^{-1} sec^{-1} (24); -12×10^{-5} cm^2 volt^{-1} sec^{-1} (25)). On acid hydrolysis substance d liberates 2-amino-2-deoxy sugars (accounting for more than 90% of the saccharide constituents), but no hexoses, hexose uronic acids, or inorganic acids. The amino sugars present were a hexosamine and an acidic amino sugar, which behaved on a paper chromatogram (Solvent B) like a hexosamine uronic acid (16). Paper electrophoresis also separated two components with similar mobilities to a hexosamine and a hexosamine uronic acid.

Ninhydrin degradation yielded arabinose, indicating that the hexosamine was glucosamine or mannosamine; however, the latter was excluded by the electrophoretic migration of the *N*-acetyl derivative. Also produced in the ninhydrin degradation was a component with chromatographic behavior which was consistent with its assignment as a pentose uronic acid, which would be expected to result from a hexosamine uronic acid.

A study of the deamination products of the amino sugars by borate electrophoresis was consistent with the presence of glucosamine and glucosamine uronic acid and the absence of galactosamine uronic acid (19).

A small sample of the hexosamine uronic acid was isolated by virtue of the insolubility of the free base in methanol (16) and the crystalline hydrochloride was identified as glucosamine uronic acid hydrochloride by its infrared spectrum.

The relative amounts of the two amino sugars suggest that, if there is a regular repeating unit, it consists of two glucosamine uronic acid residues and one glucosamine residue. The optical rotation ($[\alpha]_D$ −62.6) (McPherson *et al.* (6)) of the intact polysaccharide may indicate β-linkages and the resistance of the polymer to periodate oxidation may mean that the linkages are 1 → 3 or 1 → 4. The *N*-acetyl analysis indicates that there is more than 90% acetylation of the amino groups in the polymer.

In Table I the experimental analysis is compared to the theoretical for a polymer having as its basic unit 2 molecules of *N*-acetylglucosamine uronic acid and 1 molecule of *N*-acetylglucosamine.

TABLE I

Comparison of analysis of substance d with values calculated for structure suggested in text

	Theoretical	Experimental[a]
Glucosamine (%)	28.5	25.4
Glucosamine uronic acid (%)	61.5	51.0
Total hexosamine (%)	85.5	72.7[b]
		76.1[c]
N-Acetyl	18.9	16.3[d]

[a] Values corrected only for protein content.
[b] Sum of individual components determined (19) after column separation.
[c] Determined by deamination (10) of total acid hydrolysate.
[d] Corrected for moisture, ash, and protein content.

SUMMARY

The type-specific substance of *Hemophilus influenzae*, type d, has been isolated as a nonreducing polysaccharide and its structure studied. Substance d contains *N*-acetylglucosamine uronic acid and *N*-acetylglucosamine approximately in a 2:1 molar ratio. The linkages are tentatively assigned as 1 → 3, or 1 → 4, β.

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